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JC10 Rec'd PCT/PTO 15 MAR 2002

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371		Attorney's Docket Number 045636-5055
International Application No.	International Filing Date	U.S. Application No. Unassigned
PCT/FR00/02565	September 15, 2000	Priority Date Claimed September 17, 1999

Title of Invention: METHOD FOR GENETIC MODIFICATION OF LACTOBACILLUS DELBRUECKII

Applicants For EO/EO/US: Pascale SERROR, Golnar ILAMI-NESPOULOUS, Maarten VAN DE GUCHTE, Christian CHERVAUX, Christophe FREMAUX, Laurent BENBADIS and Emmanuelle MAGUIN

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. § 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. § 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. § 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. § 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. § 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. § 371(c)(3)).
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)).
9. ☐ An oath or declaration of the inventors (35 U.S.C. § 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. § 371(c)(5)).

Items 11. to 14. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. § 1.97 and § 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. § 3.28 and § 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☒ Other items or information:
 - a. WO 01/21819 cover sheet including English language abstract
 - b.
 - c.
 - d.
 - e.
 - f.

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U.S. APPLICATION NO. | INTERNATIONAL APPLICATION NO. | ATTORNEY DOCKET NUMBER

Unassigned

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15. ☒

☒ The following fees are submitted:

Basic National Fee (37 C.F.R. § 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO.....\$890.00

International preliminary examination fee paid to

USPTO (37 C.F.R. § 1.482).....	\$710.00
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No international preliminary examination fee paid to

USPTO (37 C.F.R. § 1.482) but international search fee

paid to USPTO (37 C.F.R. § 1.445(a)(2)).....\$740.00

Neither international preliminary examination fee

(37 C.F.R. § 1.482) nor international search fee

(37 C.F.R. § 1.445(a)(2)) paid to USPTO.....\$1,040.00

International preliminary examination fee paid to USPTO

(37 C.F.R. § 1.482) and all claims satisfied provisions

of PCT Article 33(2)-(4).....\$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT	=	\$890.00
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Surcharge of \$130.00 for furnishing the oath or declaration later than

☐ 20 ☒ 30 months from the earliest claimed priority date

(37 C.F.R. § 1.492(e)).

is

<u>Claims</u>	<u>Number Filed</u>	<u>Number Extra</u>	<u>Rate</u>	
Total Claims	10 - 20 =	0	X \$18.00	\$
Independent Claims	4 - 3 =	1	X \$84.00	\$ 84.00
Multiple dependent claim(s) (if applicable)			+ \$280.00	\$
TOTAL OF ABOVE CALCULATIONS				\$ 974.00
Reduction by ½ for filing by small entity, if applicable.				
Verified Small Entity statement must also be filed. (Note 37 C.F.R. §§ 1.9, 1.27, 1.28)				-\$
SUBTOTAL =				\$ 974.00
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(f)).				+\$
TOTAL NATIONAL FEE =				\$974.00
Fee for recording the enclosed assignment (37 C.F.R. § 1.21(h)). The Assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§ 3.230, 3.31). \$40.00 per property				\$
TOTAL FEES ENCLOSED =				\$
Amount to be refunded				\$
Amount to be charged				\$

- a. ☐ A check in the amount of \$_____ to cover the above fees is enclosed.
- b. ☒ Please charge my Deposit Account No. 50-0310 in the amount of **\$974.00**
- c. ☒ **Except** for issue fees payable under 37 C.F.R. § 1.130, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. § 1.16 and § 1.17 which may be required, or credit any overpayment to Deposit Account No. 50-0310.

Customer No. 09629

SEND ALL CORRESPONDENCE TO:

Morgan, Lewis & Bockius LLP

1111 Pennsylvania Avenue, N.W.

Washington, D.C. 20004

Telephone: (202) 739-3000

Facsimile: (202) 739-3001

Elizabeth C. Weimar
Elizabeth C. Weimar
Reg. No. 44,478

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

IN THE CLAIMS:

Add the following claims 11-20:

11. A method of introducing a modification of the genetic material of a bacterium comprising introducing a plasmid comprising the pIP501 theta replication system, or a related replication system, as a thermosensitive conditional vector for integration into a bacterium of the species *Lactobacillus delbrueckii*.

12. A method for modifying the genetic information carried by the chromosome of a bacterium of the species *Lactobacillus delbrueckii* comprising:

a) constructing an integrative plasmid by inserting at least one DNA sequence capable of integrating into the bacterial chromosome into a conditional vector comprising the pIP501 theta replication system, or a related replication system, wherein the integrative plasmid also carries at least one selection marker;

b) introducing the plasmid into a *Lactobacillus delbrueckii* bacterium and multiplying the resultant bacterium under conditions which permit the replication and maintenance of the plasmid in stable form;

c) multiplying the bacteria under conditions which prevent the replication and/or maintenance of the plasmid in stable form.

13. The method as claimed in claim 12, further comprising recovering the bacteria expressing at least one selection marker originating from the plasmid..

14. The method of claim 12 wherein the DNA sequence capable of inserting into the bacterial chromosome is a sequence homologous to a portion of the chromosome into which it is desired to introduce a modification.

15. The method of claim 12 wherein the DNA sequences capable of inserting into the bacterial chromosome is a transposable sequence.

16. The method of claim 15 wherein the transposable sequence is an insertion sequence.
17. The method of claim 16, wherein the insertion sequence is chosen from IS1223 of *Lactobacillus johnsonii* and IS1201 of *Lactobacillus helveticus*.
18. A method of modifying the chromosome of *Lactobacillus delbrueckii* comprising introducing an insertion sequence chosen from IS1223 from *Lactobacillus johnsonii* and IS1201 of *Lactobacillus helveticus* into the nucleus of *Lactobacillus delbrueckii* cells.
19. An integrative plasmid comprising one of the sequences IS1223 or IS1201 and either:
- a) the pVE6002 replication system, or
 - b) the pIP501 replicon theta replication system or a related replication system.
20. The integrative plasmid of claim 19, wherein the plasmid is chosen from the group consisting of the pVI49 plasmid and the pVI52 plasmid, deposited with the CNCM on September 17, 1999, under the respective numbers I-2317 and I-22318.

REMARKS

The changes to the claims requested above have been made so as to eliminate multiple claim dependencies and to present claim language more conventional for practice in the United States.

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METHOD FOR GENETIC MODIFICATION OF LACTOBACILLUS
DELBRUECKII

The present invention relates to the genetic
5 modification of *Lactobacillus delbrueckii*.

Lactic acid bacteria are used a great deal in the agrofoods industry, particularly for manufacturing diverse fermented products; in addition, their innocuity has made their use recommended for the production, by genetic engineering, of diverse substances intended in particular for therapeutic use.

The genetic modification of lactic acid bacteria makes it possible to adjust their characteristics depending on the use envisaged, whether by the introduction and expression of foreign DNA, or by the overexpression or, on the contrary, the inactivation of genes naturally present in said bacteria. For a modification to be stable, it must be integrated into the bacterial chromosome. With this aim, the desired modification is inserted into a nonreplicative plasmid carrying a selection marker. The vector thus obtained is introduced into the bacteria; the bacteria expressing the selection marker, which are those which have integrated the vector into their chromosome, are then recovered.

The modification results from 2 events which occur at low frequency: 1) the introduction of the plasmid into the bacteria; 2) the integration of said plasmid into the chromosome. The modification rate which may be anticipated is the product of the probabilities of these two events; it is therefore very low, especially in the case of bacteria belonging to species which are "refractory" to transformation, which is the case for many species of lactic acid bacteria. Among these, mention will be made in particular of the species

particular compound, etc.), either due to the fact that one or more of the proteins involved in this function comprise(s) one or more mutation(s) making the activity conditional, or due to the fact that one or more of the proteins involved in this function is (are) placed under the control of an inducible promoter.

BISWAS et al., J. Bacteriol., 175, 3628-3635, (1993);
MAGUIN et al., J. Bacteriol., 178, 931-935, (1996) and
10 PCT application WO 93/18164 thus describe integrative
vectors which comprise the pVE6002 mutant replicon
replication system (CNCM I-1179), also named pG⁺host,
which is a thermosensitive (Ts) derivative of the
plasmid pWV01 due to a mutation in the sequence
15 encoding the RepA protein.

The modification may thus be carried out in two steps: in the first, the plasmid is introduced into the bacteria, at permissive temperature, which allows a first selection of those in which it has installed itself; in the second, these bacteria are cultured at a temperature which is nonpermissive for the replication of the plasmid, which makes it possible to select those in which the sequences introduced are integrated into the chromosome.

PCT application WO 93/18164 describes the use of these plasmids in diverse species of lactic acid bacteria, including *Lb. delbrueckii* subsp. *bulgaricus*.

In pursuing their investigations, the inventors have presently noted that other plasmids used in lactic acid bacteria are capable of replicating in *Lb. delbrueckii* at temperatures possibly reaching approximately 37°C, which allow the growth of this species, but are thermosensitive at higher temperatures, generally from 42°C, which is the optimum temperature for growth of this species.

thermosensitivity in *Lb. delbrueckii*, of pIP501. This encompasses in particular any theta replication system comprising:

- 5 - an origin of replication, *ori*, having at least 80% identity, preferably at least 85% identity and advantageously at least 90% identity, at the level of the nucleotide sequence, with the origin of replication, *ori*, of pIP501 and/or;
- 10 - a sequence encoding a *rep* protein, the peptide sequence of which has at least 85% identity, preferably at least 90% identity, and advantageously at least 95% identity, with that of the *repR* protein of pIP501.

15 The sequence of the origin of replication, *ori*, of
pIP501 is in particular described by BRANTL and BEHNKE
[Molecular Microbiology, 6, 3501-3510 (1992)]; the
sequence encoding the *repR* of pIP501 is in particular
identified in the complete sequence of pGB3631 (derived
20 from pIP501), published by BRANTL et al., [Gene, 142,
155-156, (1994)], and accessible under the number
X72021.

25 The percentage identity of a sequence with a reference sequence is defined herein as the percentage of residues of this sequence which are identical to those of the reference sequence over an alignment of the 2 sequences which ensures that the positions of the residues correspond to a maximum.

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A subject of the present invention is in particular a process for modifying the genetic information carried by the chromosome of a bacterium of the species *Lb. delbrueckii*, characterized in that it comprises:

- 35 a) constructing an integrative plasmid by inserting
at least one DNA sequence, capable of integrating
into the bacterial chromosome, into a conditional
vector comprising the pIP501 theta replication
system, or a related replication system, said

b) introducing the plasmid into said bacterium and multiplying this bacterium, under conditions permissive for the replication and maintenance in stable form of said plasmid;

d) recovering the bacteria expressing at least one selection marker originating from the plasmid, at the end of step c).

According to a variant of the process in accordance with the invention, the step of multiplying the bacterium under conditions permissive for the replication and maintenance of the plasmid in stable form may be omitted; in this case, after introducing the plasmid into the bacterium, the multiplication thereof is carried out directly under conditions nonpermissive for the replication and/or maintenance in stable form of the plasmid.

Advantageously, it is possible, after having obtained bacteria carrying the desired modification, to remove the sequences originating from the vector used in step a), in cases in which it is not desirable to keep these sequences, for example in the case of bacteria intended for industrial use, in particular for the preparation of foodstuffs.

In this case, the process in accordance with the invention also comprises the following steps:

e) excising the sequences originating from the vector, by multiplying the bacteria expressing, at the end of step c), at least one selection marker originating from the plasmid and, advantageously,

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Another possibility for selection at the end of step c) consists in using a property of the strain which ensues

from the integration of the plasmid and/or from the excision of the vector sequences. For example, use may be made of a conditional plasmid comprising a selection marker M (for example a resistance marker) and a DNA sequence capable of integrating by homologous recombination into a bacterial gene X, said gene X being essential under certain conditions for the bacterium (for example a gene essential for the growth thereof on a given medium), the integration of the plasmid inactivating the gene X, and the excision of the sequences of the vector restoring its activity. In this case, the bacteria in which the plasmid DNA has been integrated may be selected on the basis of the expression of the selection marker M, under conditions in which the gene X is not essential, and the bacteria in which the sequences of the integrated vector have been excised may be selected, under conditions in which the gene X is essential, on the basis of the restoration of its activity. In addition, this method of selection also makes it possible to select, in a single step, bacteria in which the integration of the plasmid DNA and the excision of the sequences of the vector have taken place; in this case, the bacteria are directly cultured under conditions in which the gene X is essential, and which are also selective for the marker M. The bacteria selected will be those in which the integration of the plasmid and the excision of the sequences of the vector (including the marker M) have restored the activity of the gene X.

DNA sequences capable of integrating into the chromosome of a bacterium of the species *Lb. delbrueckii* in particular comprise:

- sequences chosen on the basis of their homology with a portion of the chromosome into which it is desired to introduce a modification, i.e. exhibiting a homology with this portion of the chromosome which is sufficient to be able to recombine with it;

- transposable sequences, in particular transposons or insertion sequences (IS); such sequences may insert into the bacterial chromosome randomly or with a certain specificity.

The modifications intended to be integrated into the bacterial chromosome may be introduced by the simple integration of these sequences, this integration possibly, for example, leading to the inactivation of a gene within which it occurs. It is also possible to use these sequences to integrate, into the bacterial chromosome, a DNA fragment, in particular a gene of interest of heterologous origin, or a DNA fragment from *Lb. delbrueckii*, modified beforehand.

In the case of the integration by homologous recombination, for example, it is possible to introduce, into a sequence identical to that of the region of the chromosome intended to be modified, modifications by insertion, deletion or substitution which may range from a single nucleotide to several thousands of nucleotides. The sequence thus modified is inserted in step a) of the process according to the invention, into a vector which is conditional in *Lb. delbrueckii*, and which comprises the pIP501 theta replication system, or a related system, as defined above. The integration takes place by recombination (simple crossing-over) between the cloned chromosomal DNA fragment and the homologous region of the bacterial chromosome. Since this recombination event creates duplications of the region of homology, on both sides of the vector sequences, the structure integrated into the chromosome consists of the vector sequences bordered on both sides by a copy of the region of homology, as shown in figure 1.

The use of sequences which can integrate by homologous recombination in particular makes it possible:

- 10 -

- to inactivate one (or more) bacterial gene(s);
- to modify the expression of the genes and/or the activity of the products encoded by these genes;
- to introduce, in a stable manner, new functions into the chromosome;
- to study and to use the expression of the genes *in situ*;
- to mutate the chromosome by integration via random chromosomal fragments;
- to insert, in a stable manner, sequences intended to label the strains. These sequences will subsequently be used as tracers;
- to introduce several sequential modifications into the same strain.

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In the case of the integration via a transposable sequence, the transposable sequence will be inserted into the plasmid; the integration into the chromosome makes it possible to produce the modification, to thus obtain mutants exhibiting advantageous characteristics, and to characterize more easily the mutated gene(s). The structure transposed into the chromosome may consist of the vector sequences bordered on both sides by a copy of the transposable sequence.

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These transposable sequences may originate from bacteria belonging to the species *Lb. delbrueckii*, or originate from other bacterial species, in particular other lactic acid bacteria. They may be transposons or insertion sequences.

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Transposable sequences which are functional in *Lb. delbrueckii* may be identified by inserting a transposable sequence to be tested (transposon or insertion sequence) into a vector which replicates conditionally in *Lb. delbrueckii* and which comprises the pIP501 theta replication system, or a related system, as defined above, by carrying out steps b) and c) of the process in accordance with the invention and

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searching for the presence of transposants at the end of these steps.

The inventors have thus demonstrated 2 insertion sequences which are functional in *Lb. delbrueckii*. A subject of the present invention is also the use of either of these sequences for modifying the chromosome of *Lb. delbrueckii*.

They are the insertion sequence named IS1233, previously demonstrated by WALKER et al. (J. Bacteriol., 176, 5330-5340, 1994) in *Lb. johnsonii*, and the insertion sequence named IS1201, previously demonstrated by TAILLIEZ et al. (Gene, 145, 75-79, 1994) in *Lb. helveticus*.

A subject of the present invention is also any integrative plasmid resulting from the insertion of one of these 2 sequences into a vector which replicates conditionally in *Lb. delbrueckii*, and in particular into a vector comprising the pVE6002 replication system, such as those described in PCT application WO 93/18164, or into a vector comprising the pIP501 theta replication system, or a related system, as defined above.

According to a preferred embodiment of the present invention, said vector is a nonconjugative vector.

Integrative plasmids in accordance with the invention are in particular illustrated by the pVI49 plasmid and the pVI52 plasmid. The pVI49 plasmid harbored by the *E. coli* strain VI209, and the pVI52 plasmid harbored by the *E. coli* strain VI217, were deposited, according to the Treaty of Budapest, on September 17, 1999, under the respective numbers I-2317 and I-2318, with the CNCM (Collection Nationale de Cultures de Microorganismes [National Collection of Microorganism Cultures]), 25 rue du Docteur Roux, Paris.

NP: nonpermissive conditions.

The bacteria selected at nonpermissive temperature during step c) ($>42^{\circ}\text{C}$) are cultured without any selection pressure so as to allow recombination between the homologous regions flanking the vector sequences.

After removal of the excised sequences, in accordance with step f) of the process in accordance with the invention, the following are thus obtained:

- in the case of a plasmid comprising a sequence capable of integrating by homologous recombination, a bacterium which differs from the host bacterium of origin by the presence, in its chromosome, of the modification introduced by this sequence;
- in the case of a plasmid comprising a transposable sequence, a bacterium which differs from the host bacterium of origin by the presence, in its chromosome, of a copy of said transposable sequence.

The present invention will be more fully understood with the aid of the further description which follows, 25 which refers to nonlimiting examples of the production of integrative plasmids and of their use in *Lb. delbrueckii*.

30 EXAMPLE 1: SELECTION OF PLASMIDS WHICH ARE
 THERMOSENSITIVE IN *Lb. delbrueckii*

Production of plasmids

An *L. bulgaricus* - *E. coli* shuttle plasmid consisting of pGB3631 (a sequence derivative of pIP501, (BRANTL et al., Gene, 142, 155-156, (1994)]) and of the pSKII origin of *E. coli* was constructed. This plasmid, named pVI1055, is represented in figure 3. It carries an ery gene for erythromycin (Ery) resistance.

5 Demonstration of the thermosensitivity in *Lb.*
delbrueckii

10 Bacteria of the *Lb. delbrueckii* subsp. *bulgaricus*
strain ATCC 11842 or of the strain VI104 deposited
according to the Treaty of Budapest, on September 17,
1999, under the number I-2316, with the CNCM
(Collection Nationale de Cultures de Microorganismes
15 [National Collection of Microorganism Cultures]),
25 rue du Docteur Roux, Paris, are cultured in MRS
medium containing 0.1% glycine (DIFCO), until the
beginning of the stationary phase. They are then
centrifuged and washed in electroporation buffer (0.4 M
20 sucrose, 1 mM MgCl₂, 5 mM KH₂PO₄, pH 6.0) and suspended
in this same buffer at a concentration corresponding to
an OD₆₀₀ of approximately 50. The suspension is
incubated for 20 minutes at 45°C and then cooled on
ice. An 80 µl aliquot of the suspension is mixed with
25 the plasmid DNA (~1.5 µg) and the mixture is
transferred into a 0.2 cm electroporation cuvette. The
electroporation is performed at 1 kV, 800 Ω, and 25 µF.

Immediately after electroporation, the mixture is
30 diluted in 2 ml of expression medium (0.2 M sucrose, 5%
powdered skimmed milk, 0.1% yeast extract, 1% casamino
acids, 25 mM $MgCl_2$). After incubation for 3 h at 37°C
in this medium, the cells are plated out onto dishes
containing selective medium (MRS agar with 10 µg/ml of
35 erythromycin); the dishes are incubated in anaerobic
jar at 37°C for 48 h, and the erythromycin-resistant
colonies are selected.

Thermosensitivity of pIP501 derivatives (theta replication)

The thermosensitivity of pGB305Δ and of pVII1055 was first evaluated using *Lb. delbrueckii* (ATCC 11842 or 5 VII104) transformation assays according to the protocol described above, with plating out onto selective medium at 37°C and 42°C. For both plasmids, transformants are obtained at 37°C but not at 42°C.

10 The thermosensitivity of pVI1055 was then confirmed by comparing its stability at 37°C and at 44°C. A culture in MRS/Ery (10 µg/ml) at 37°C was diluted in MRS without Ery, and then incubated in parallel at 37°C and
15 at 44°C. Samples are taken at various times and the cells are plated out after dilution on MRS and MRS/Ery, in order to determine the proportion of cells having lost the plasmid.

20 At 37°C, approximately 30% of the cells have lost the pVI1055 plasmid after 48 hours. At 44°C, the stability of pVI1055 is clearly affected, since ~100% of the cells have lost the plasmid after 48 hours.

25 **EXAMPLE 2: DEMONSTRATION OF THE TRANSPOSITION ACTIVITY**
 OF INSERTION SEQUENCES (IS) IN *Lb. delbrueckii*

Insertion sequences isolated from lactic acid bacteria were cloned into pVI1055. At a temperature permissive for the replication of the plasmid, the multiplication of a transformant generates a bacterial population containing the plasmid. The IS present on the plasmid in the bacterial population may transpose into the chromosome. In the event of transposition of the IS, the transposed structure present in the chromosome may correspond to the plasmid vector bordered, on both sides, by a copy of the IS, as shown in figure 4. At nonpermissive temperature (44°C), the plasmids containing the IS lead to the production of Ery^R clones

with a frequency greater than that observed for pVI1055, the plasmid without IS. In the case of the production of Ery^R clones, structural analysis of the chromosomal DNA by Southern Blot with a probe
5 corresponding to the IS tested makes it possible to verify that the integration indeed results from a transposition event.

Demonstration of the transposition:

10 The IS 1223 (WALKER and KLAENHAMMER, 1994,
abovementioned reference) and 1201 (TAILLEZ et al.,
1994, abovementioned reference) were cloned into the
pVI1055 plasmid, generating, respectively, the plasmids
pVI48, pVI49 (corresponding to the 2 orientations of
15 IS1223) and pVI52.

The properties of these 2 IS and the nature of the plasmids obtained are indicated in table 1 below.

TABLE 1

IS	Origin	Size ^a (bp)	Final vectors ^b
1223	<i>L. johnsonii</i>	1502	pVI48/pVI49
1201	<i>L. helveticus</i>	1387	pVI52

20 a: size corresponding to the IS bordered by the direct
repeats (DR)

b: when two plasmids are given, this indicates that the IS was cloned in both orientations

25 In order to search for the transposition activity, the strains containing the plasmids carrying the IS to be tested were cultured at 37°C in MRS/Ery (10 µg/ml). The cells are then diluted in MRS and incubated at 44°C. Samples were taken at various times and plated out on
30 MRS dishes (to determine the viable cell count) and on MRS/Ery (Ery^R cell count).

Results:

IS1223 (pVI48/pVI49): the pVI48 or pVI49 plasmid was
35 introduced into VI104. In the presence of IS1223, the
frequency of Ery^R cells is higher than with pVI1055
alone. After digestion, the chromosomal DNA of the Ery^R

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clones obtained are hybridized with IS1223. In all cases, the Ery^R clones are the result of transposition events.

- 5 **IS1201 (pVI52):** the pVI52 plasmid was introduced into VI104. The frequency of integration of pVI52 is higher than that of pVI1055 alone. All the Ery^R clones analyzed by Southern are the result of transposition.
- 10 These results illustrate the transposition activity of IS 1223 and 1201 in *Lb. delbrueckii*.

CLAIMS

1. The use of a plasmid comprising the pIP501 theta
replication system, or a related replication
5 system, as a thermosensitive conditional vector
for integration which makes it possible to
introduce a modification of the genetic
information of a bacterium of the species
Lb. delbrueckii.
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2. A process for modifying the genetic information
carried by the chromosome of a bacterium of the
species *Lb. delbrueckii*, characterized in that it
comprises:
15 a) constructing an integrative plasmid by
inserting at least one DNA sequence, capable of
integrating into the bacterial chromosome, into
a conditional vector comprising the pIP501
theta replication system, or a related
20 replication system, said integrative plasmid
also carrying at least one selection marker;
b) introducing the plasmid into said bacterium and
multiplying this bacterium, under conditions
permissive for the replication and maintenance
25 in stable form of said plasmid;
c) multiplying the bacteria expressing at least
one selection marker from the plasmid at the
end of step b), under conditions nonpermissive
for the replication and/or maintenance in
30 stable form of said plasmid; and, optionally,
d) recovering the bacteria expressing at least one
selection marker originating from the plasmid,
at the end of step c).
- 35 3. The process as claimed in claim 2, characterized
in that it also comprises:
e) excising the sequences originating from the
vector, by multiplying the bacteria expressing,

at the end of step c), at least one selection marker originating from the plasmid and, advantageously,

5 f) removing the excised DNA by multiplying the bacteria obtained in step e), under conditions nonpermissive for the replication and maintenance of said vector in stable plasmid form.

10 4. The process as claimed in either of claims 2
and 3, characterized in that the DNA sequence
capable of inserting into the bacterial chromosome
is a sequence homologous to a portion of the
chromosome into which it is desired to introduce a
15 modification.

5. The process as claimed in either of claims 2 and 3, characterized in that the DNA sequence capable of inserting into the bacterial chromosome is a transposable sequence.

6. The process as claimed in claim 5, characterized in that said transposable sequence is an insertion sequence.

7. The process as claimed in claim 6, characterized in that said insertion sequence is chosen from IS1223 of *Lb. johnsonii* and IS1201 of *Lb. helveticus*.

8. The use of an insertion sequence chosen from IS1223 from *Lb. johnsonii* and IS1201 of *Lb. helveticus*, for modifying the chromosome of *Lb. delbrueckii*.

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9. An integrative plasmid for carrying out a process as claimed in any one of claims 1 to 6, characterized in that it results from the insertion of one of the sequences IS1223 or IS1201

- 20 -

into a plasmid chosen from:

- the plasmids comprising the pVE6002 replication system;
- the plasmids comprising the pIP501 replicon theta replication system, or a related replication system.

10. The integrative plasmid as claimed in claim 8,
chosen from the group consisting of the pVI49
10 plasmid and the pVI52 plasmid, deposited with the
CNCM on September 17, 1999, under the respective
numbers I-2317 and I-2318.

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(71) Déposants (pour tous les États désignés sauf US):
INSTITUT NATIONAL DE LA RECHERCHE
AGRONOMIQUE (INRA) [FR/FR]; 147, rue de l'Uni-
versité, F-75007 Paris (FR). COMPAGNIE GERVAIS
DANONE [FR/FR]; 126-130, rue Jules Guesde, F-92302
Levallois-Perret (FR). RHODIA FOOD [FR/FR]; 25,
quai Paul Doumer, F-92408 Courbevoie (FR).

(72) Inventeurs; et

(75) Inventeurs/Déposants (pour US seulement): SER-
ROR, Pascale [FR/FR]; 6, rue des Coloristes, F-78350
Jouy-en-Josas (FR). ILAMI-NESPOULOUS, Golnar
[—/FR]; 53, allée de la Ferme d'Armenon, F-91190
Gif-sur-Yvette (FR). VAN DE GUCHTE, Maarten
[—/FR]; 9, square Raphaël, F-78150 Le Chesnay (FR).
CHERVAUX, Christian [FR/FR]; 31, rue de la Bièvre,
F-78350 Buc (FR). FREMAUX, Christophe [FR/FR];

19, rue des Joncs, F-86000 Poitiers (FR). BENBADIS,
Laurent [FR/FR]; 7, avenue de Provence, F-92160 Antony
(FR). MAGUIN, Emmanuelle [FR/FR]; 16, avenue du
Fort, F-92150 Montrouge (FR).

(74) Mandataires: VIALLE-PRESLES, Marie-José etc.;
Cabinet Orés, 6, avenue de Messine, F-75008 Paris (FR).

(81) États désignés (national): AE, AG, AL, AM, AT, AU, AZ,
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(54) Title: METHOD FOR GENETIC MODIFICATION OF LACTOBACILLUS DELBRUECKII

(54) Titre: PROCEDE DE MODIFICATION GENETIQUE DE LACTOBACILLUS DELBRUECKII

(57) Abstract: The invention concerns a method for modifying the chromosomal genetic information of *Lactobacillus delbrueckii*, using a conditional integrator plasmid. The invention also concerns integrator plasmids for use in implementing said method.

(57) Abrégé: L'invention concerne un procédé de modification de l'information génétique chromosomique de *Lactobacillus delbrueckii*, mettant en oeuvre un plasmide intégratif conditionnel. L'invention concerne également des plasmides intégratifs utilisables pour la mise en oeuvre dudit procédé.

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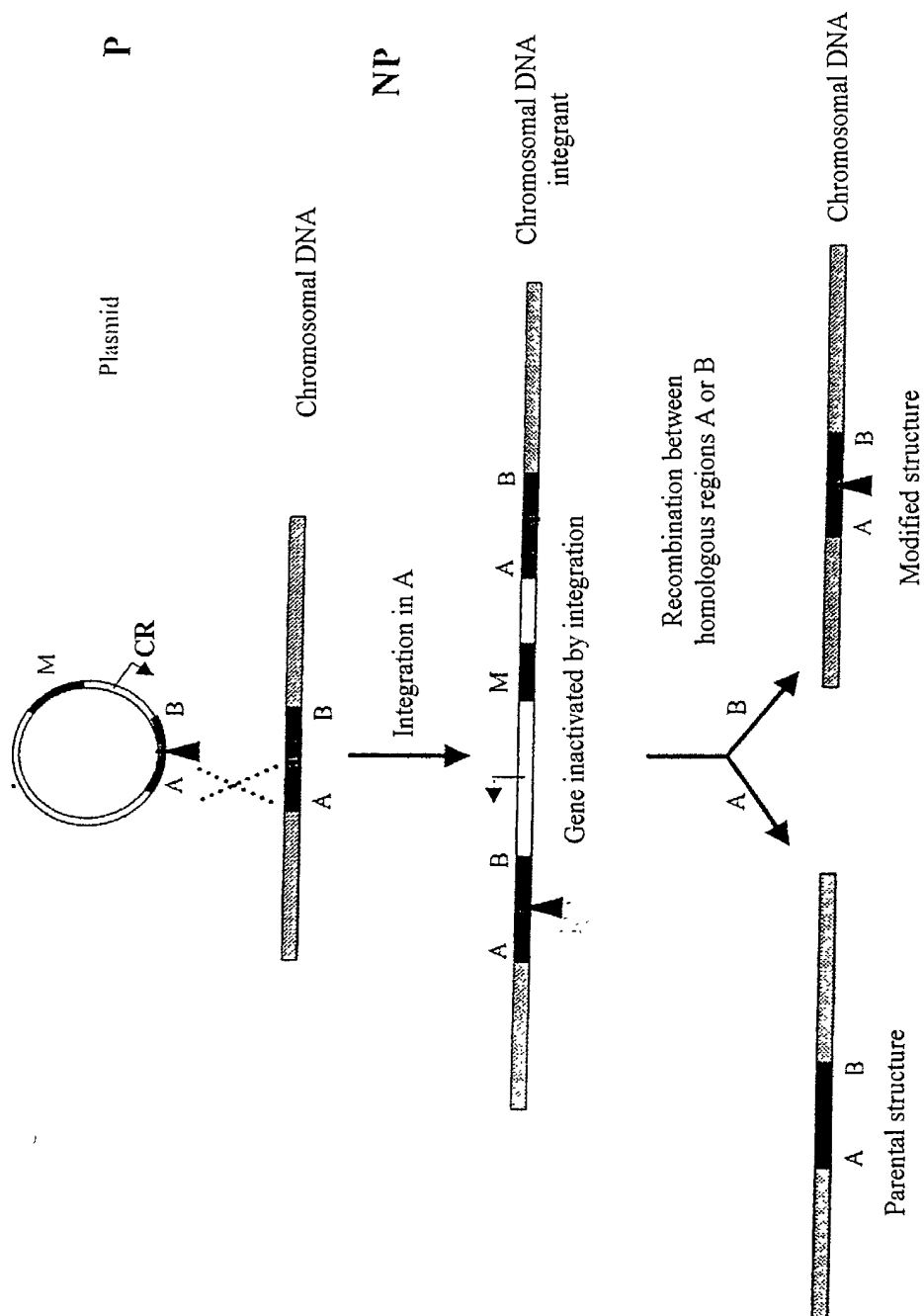


Figure 1

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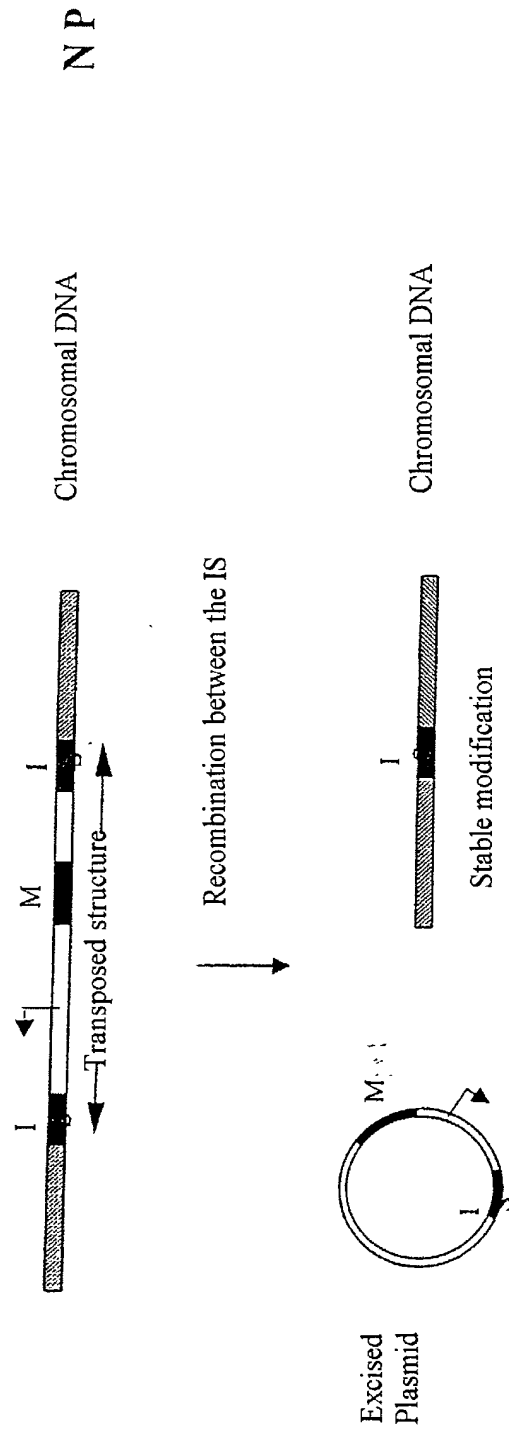
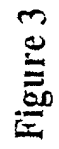


Figure 2

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#4

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METHOD FOR GENETIC MODIFICATION OF *LACTOBACILLUS DELBRUEKII*

is attached hereto ; or

was filed as a PCT international application Number PCT/FR00/02565 on September 15, 2000 and was amended under PCT article 19 on _____ (if applicable).

I acknowledge the duty to disclose the U.S. Patent and Trademark Office information which is material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, § 1.56.

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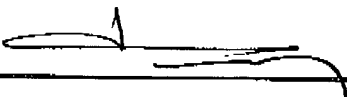
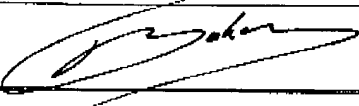
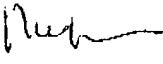
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Customer Number: 009629

Direct Telephone Calls To :
(name and telephone number)

5-00	FULL NAME OF FIFTH INVENTOR	FREMAUX Christophe	
	RESIDENCE & CITIZENSHIP	86000 <u>POITIERS</u> <i>FRX</i>	COUNTRY OF CITIZENSHIP FRANCE
	POST OFFICE ADDRESS	19, Rue des Joncs 86000 POITIERS FRANCE	
	FIFTH INVENTOR'S SIGNATURE		DATE 2 Aout 2002 .
6-00	FULL NAME OF SIXTH INVENTOR	BENBADIS Laurent	
	RESIDENCE & CITIZENSHIP	92160 <u>ANTONY</u> <i>FRX</i>	COUNTRY OF CITIZENSHIP FRANCE
	POST OFFICE ADDRESS	7, Avenue de Provence 92160 ANTONY FRANCE	
	SIXTH INVENTOR'S SIGNATURE		DATE 18/7/02
7-00	FULL NAME OF SEVENTH INVENTOR	MAGUIN Emmanuelle	
	RESIDENCE & CITIZENSHIP	92120 <u>MONTROUGE</u> <i>FRX</i>	COUNTRY OF CITIZENSHIP FRANCE
	POST OFFICE ADDRESS	16, Avenue du Fort 92120 MONTROUGE FRANCE	
	SEVENTH INVENTOR'S SIGNATURE		DATE 6/7/02
	FULL NAME OF EIGHTH INVENTOR		
	RESIDENCE & CITIZENSHIP		COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS		
	EIGHTH INVENTOR'S SIGNATURE		DATE
	FULL NAME OF NINTH INVENTOR		
	RESIDENCE & CITIZENSHIP		COUNTRY OF CITIZENSHIP
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